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PNA Targeting the PBS and A-Loop Sequences of HIV-1 Genome Destabilizes Packaged $tRNA_3^{Lys}$ in the Virions and Inhibits HIV-1 Replication

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During assembly of the HIV-1 virions, cellular $tRNA_3^{Lys}$ is packaged into the virion particles and is utilized as a primer for the initiation of reverse transcription. The 3′-terminal 18 nucleotides of the cellular $tRNA_3^{Lys}$ are complementary to nucleotides 183–201 of the viral RNA genome, referred to as the primer binding sequence (PBS). Additional sequences (A-Loop) upstream of the PBS are essential for tRNA primer selection. We report here that a PNA targeted to PBS and A-Loop sequence (PNA_{PBS}) exhibits high specificity for its target sequence and prevents $tRNA_3^{Lys}$ priming on the viral genome. We also demonstrate that PNA_{PBS} is able to invade the duplex region of the $tRNA_3^{Lys}$–viral RNA complex and destabilize the priming process, thereby inhibiting the *in vitro* initiation of reverse transcription. The endogenously packaged $tRNA_3^{Lys}$ bound to the PBS region of the viral RNA genome in the HIV-1 virion is efficiently competed out by PNA_{PBS}, resulting in near complete inhibition of initiation of endogenous reverse transcription. Examination of the effect of PNA_{PBS} on HIV-1 production in CEM cells infected with pseudotyped HIV-1 virions carrying luciferase reporter exhibited dramatic reduction of HIV-1 replication by nearly 99%. Analysis of the mechanism of PNA_{PBS}-mediated inhibition indicated that PNA_{PBS} interferes at the step of reverse transcription. These findings suggest the antiviral efficacy of PNA_{PBS} in blocking the process of HIV-1 replication.

INTRODUCTION

Replication of human immunodeficiency virus type 1 (HIV-1) occurs through a multistep reverse transcription process, in which the single-stranded viral RNA genome is converted into double-stranded proviral DNA intermediate prior to integration into the host chromosome. The process of reverse transcription is carried out by the virally encoded reverse transcriptase, which utilizes the cellular $tRNA_3^{Lys}$ bound to the primer-binding site (PBS) near the 5′ nontranslated region of the viral RNA genome as primer. Among the currently utilized therapies for AIDS are inhibitors which block the process of reverse transcription of viral RNA into double-stranded DNA (Larder, 1993; Wei *et al.,* 1995; Ho *et al.,* 1995). However, the rapid emergence of drug-resistant strains has considerably overshadowed the benefits of anti-HIV-1 drugs. Selection of the dominant, preexisting drug-resistant variants and the abundance of latently infected cells (which possess the integrated proviral DNA) are the potential barriers encountered against effective drug therapy (Ho *et al.,* 1995; Wei *et al.,* 1995; Embretson *et al.,* 1993).

In recent years, it has become apparent that identification of novel viral targets and antiviral agents are needed to empower anti-HIV-1 therapeutic strategies. The unique 5′ (U5) nontranslated region (1–333 nucleotides) of the HIV-1 genome comprises a number of regulatory regions essential for viral replication which may be potential targets for drug intervention. These critical domains are as follows: (i) the primer-binding site (nucleotides 183–201), essential for $tRNA_3^{Lys}$-primed initiation of reverse transcription (Muesing *et al.,* 1987; Ratner *et al.,* 1985; Sherman *et al.,* 1992); (ii) the A-loop region (nucleotides 168–173), located upstream of the PBS and essential for the selection and interaction of $tRNA_3^{Lys}$ primer (Wakefield *et al.,* 1996; Li *et al.,* 1997); (iii) the LTR sequences at the 5′ and 3′ ends, essential for viral transcription and integration (Vink *et al.,* 1991); and (iv) the *trans*-activation response element (TAR), essential for viral gene expression via transcriptional activation (Cullen and Green, 1990) and probably having some additional role in the initiation of reverse transcription (Harrich *et al.,* 1997).

Several approaches have been used to arrest retroviral replication or regulate its gene expression. Prominent among these have been antisense strategies to block various viral replication steps, in which nucleic acids may play an important role (Helene, 1997; Leeds *et al.,* 1997). One promising approach has been the use of peptide nucleic acid (PNA) oligomers that bind selectively to complementary DNA or RNA sequences and inhibit translation and replication. PNA are nucleic acid analogues containing polynucleobase linked with peptide backbone instead of the sugar-phosphate backbone (Nielsen *et al.,* 1991). Using this approach, we have

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earlier shown that a PNA targeted to the PBS region of the viral genome blocks reverse transcription of U5 PBS HIV-1 RNA primed with the DNA primer or synthetic $tRNA_3^{Lys}$ (Lee *et al.,* 1997). We have also demonstrated that PNA targeted to the TAR sequences of viral RNA genome is able to prevent Tat–TAR interaction by efficient sequestration of the TAR and blocking the Tat-mediated transactivation of the HIV-1 LTR transcription (Mayhood *et al.,* 2000).

In this article, we report that a 16-mer anti-PBS PNA (PNA_{PBS}), targeting five nucleotides of the PBS sequences and 11 nucleotides upstream of PBS in the 5′ nontranslated region of the viral genome, can specifically sequester its target sequence and prevent $tRNA_3^{Lys}$ priming on the viral genome. Further, PNA_{PBS} is able to compete out the packaged $tRNA_3^{Lys}$, thereby destabilizing it and inhibiting the endogenous reverse-transcription process in the virions. PNA_{PBS} also dramatically inhibits HIV-1 replication in CEM cells infected with pseudotyped HIV-1 virions by predominantly interfering with the reverse-transcription process. Scrambled PNA has no influence either on the reverse transcription process or on the replication of the HIV-1-infected cells, thus indicating the specificity of PNA_{PBS}. These findings suggest the potential of PNA_{PBS} in blocking the process of HIV-1 replication.

RESULTS

Binding specificity of PNA_{PBS} to U5 PBS HIV-1 RNA

We used the labeled U5 PBS HIV-1 RNA to determine the binding specificity of PNA_{PBS} targeted to the PBS region of HIV-1 genome. The sequence of U5 PBS RNA as well as PNA_{PBS} and scrambled PNA are shown in Fig. 1. The PNA_{PBS} and scrambled PNA contain 16 and 17 bases, respectively, linked with polyamide backbone. The sequence of PNA_{PBS} was designed such that five of its N-terminal bases are complementary to the 3′ terminal PBS bases spanning nucleotides 183–187, while the remaining C-terminal bases are complementary to the sequences upstream of the PBS (172–182 nucleotides). To ascertain the ability of PNA_{PBS} to interact with PBS sequences on the viral RNA, gel-retardation assays were performed with ^{32}P-labeled 200-base-long U5 PBS RNA and varying concentrations of PNA_{PBS} or scrambled PNA (Fig. 2). Titration of PNA_{PBS} with U5 PBS RNA at molar ratios of PNA_{PBS} to U5 PBS RNA less than 1 resulted in a stoichiometric band shift of the labeled U5 PBS RNA (Fig. 2, lanes 2–10). As seen in the figure, at molar ratios of 0.1, 0.3, 0.5, 0.6, 0.7, 0.8, and 0.9 of PNA_{PBS} to labeled U5 PBS RNA, the extent of band shift corresponded to 12, 27, 52, 58, 67, 72, and 87%, respectively. A complete shift in the mobility was achieved at equimolar or molar excess of PNA_{PBS} to U5 PBS RNA (lanes 9 and 10). A similar titration was carried out in the presence of scrambled PNA. Scrambled PNA at fivefold molar excess of U5 PBS RNA exhibited no shift in the mobility of the labeled U5 PBS RNA (Fig. 2, lanes 11–15). This indicates that the gradient of band shift observed as a function of PNA_{PBS} concentration is due to sequence-specific interaction between PNA_{PBS} and U5 PBS RNA.

Disruption of binding interaction between $tRNA_3^{Lys}$ and U5 PBS RNA by PNA_{PBS}

To determine the effect of PNA_{PBS} on the formation ($tRNA_3^{Lys}$-viral RNA) complex as well as on (HIV-1 RT-$tRNA_3^{Lys}$) complex, we carried out gel-retardation analysis (Fig. 3A). Incubation of U5 PBS RNA with the labeled $tRNA_3^{Lys}$ resulted in the formation of a distinct complex between the $tRNA_3^{Lys}$ and viral RNA as discerned by a shift in their mobility on a non-denaturing gel (lane 1). Incubation of this preformed ($tRNA_3^{Lys}$–viral RNA) complex with increasing concentrations of PNA_{PBS} resulted in the disruption of this complex in a concentration-dependent manner as noted by a significant decrease in the intensity of the slower migrating complex (lanes 2–4). This suggests that the chargeless PNA_{PBS} is able to invade the $tRNA_3^{Lys}$-primed duplex region of the viral RNA resulting in the $tRNA_3^{Lys}$ displacement. This complex formation was completely blocked when PNA_{PBS} was incubated with the U5 PBS RNA prior to its priming with the $tRNA_3^{Lys}$, suggesting that PNA_{PBS} once bound to its target sequence prevents $tRNA_3^{Lys}$ priming/binding (lanes 6–8). Interestingly, an oligonucleotide having identical sequence as PNA_{PBS} was unable to disrupt the preformed complex of ($tRNA_3^{Lys}$-U5 PBS RNA), although it was able to block $tRNA_3^{Lys}$ priming when preincubated with the U5 PBS RNA prior to priming with $tRNA_3^{Lys}$ (results not shown). Incubation of the $tRNA_3^{Lys}$ with HIV-1 RT, in the absence of PNA_{PBS} (lane 9) or in its presence (lane 10) resulted in complete supershift in the mobility of the HIV-1 RT-$tRNA_3^{Lys}$-binary complex, thus suggesting that PNA_{PBS} has no influence on the binding of $tRNA_3^{Lys}$ with the viral enzyme.

A similar experiment was carried out in the presence of scrambled PNA (Fig. 3B). As shown in the figure, scrambled PNA neither disrupted the preformed complex of $tRNA_3^{Lys}$-U5 PBS RNA (lanes 2–4) nor prevented the formation of this complex when preincubated with the U5 PBS RNA prior to its priming with $tRNA_3^{Lys}$ (lanes 6–8). Similar to PNA_{PBS}, scrambled PNA did not affect $tRNA_3^{Lys}$ binding to HIV-1 RT (lanes 9 and 10). These experiments clearly indicate the potential of PNA_{PBS} in efficiently disrupting the (viral RNA–$tRNA_3^{Lys}$) complex.

Blockage of reverse transcription as a function of PNA_{PBS} concentration

Since initiation of HIV-1 reverse transcription requires the priming of HIV-1 genome with the natural $tRNA_3^{Lys}$ primer, we examined the influence of PNA_{PBS} on the initiation process of reverse transcription. For this pur-

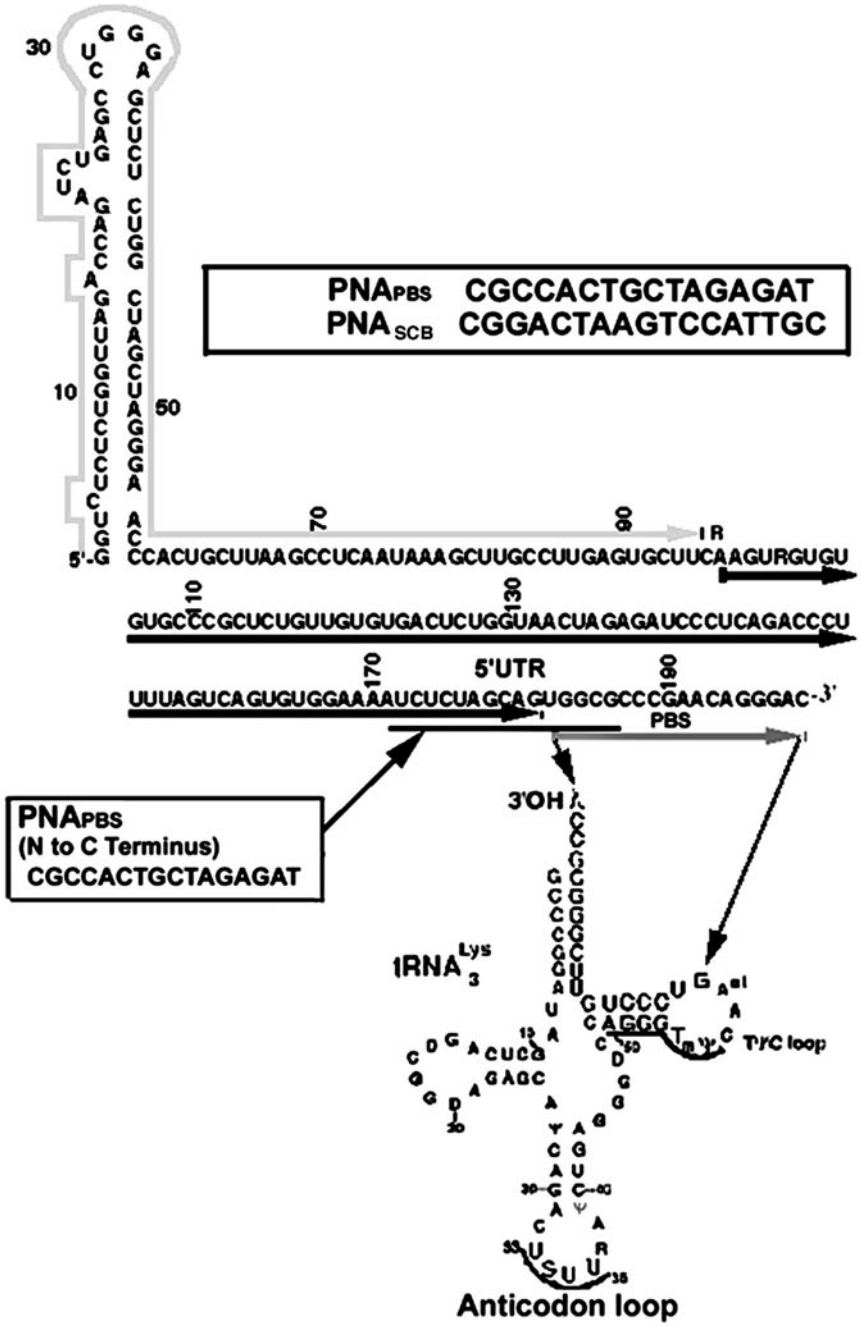

FIG. 1. Sequences of PNA_{PBS}, PNA_{SCB}, and the U5 PBS HIV-1 RNA. The sequence of U5 PBS HIV-1 RNA genome encompassing the TAR (R), 5′ UTR, and PBS regions is as shown. The sequence of PNA_{PBS} and PNA_{SCB} is as shown in the box. The 3′ terminal 18 nucleotides of $tRNA_3^{Lys}$ which primes to the PBS region are as indicated. The sequences within the PBS and 5′ UTR interacting with the PNA_{PBS} are underlined.

pose, U5 PBS RNA primed with the natural $tRNA_3^{Lys}$ was incubated at varying molar ratios of PNA_{PBS} or scrambled PNA to TP. Reverse transcription was initiated by HIV-1 RT in the presence of labeled dNTPs. The results are shown in Fig. 4A. Reverse transcription of $tRNA_3^{Lys}$-primed reaction resulted in the synthesis of (−)-strand strong stop DNA (sssDNA) of expected length (257 nucleotide) (Fig. 4A, lane 1). Incubation of the template primer with PNA_{PBS} significantly decreased the synthesis of sssDNA as a function of PNA_{PBS} concentrations (lanes 2–7). PNA_{PBS} invaded the duplex region of the template primer and displaced $tRNA_3^{Lys}$ from the viral genome, thereby inhibiting initiation as well as elongation of reverse transcription at all concentrations. Scrambled PNA had no influence on the reverse transcription under identical conditions (lanes 8–10).

In another set of experiments, the PBS sequence of U5 PBS RNA was blocked by preincubating with PNA_{PBS} and then allowed to interact with $tRNA_3^{Lys}$ prior to initiation of reverse transcription. The results shown in Fig. 4B

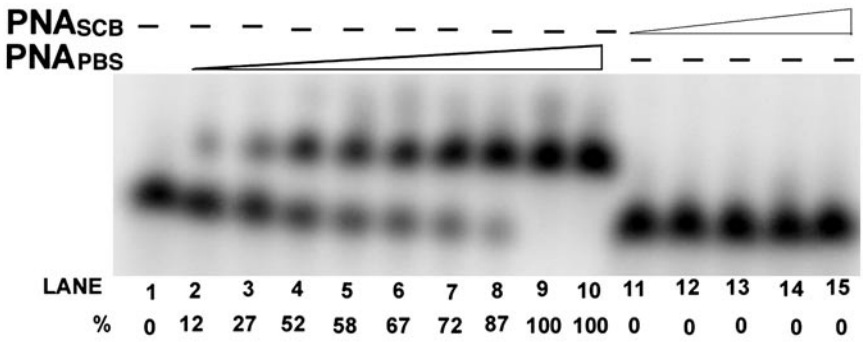

FIG. 2. Binding specificity of PNA_{PBS} to its target sequence on the HIV-1 genomic RNA. Varying molar ratios of PNA_{PBS} or PNA_{SCB} were incubated with the labeled U5 PBS RNA transcript (5×10^3 Cerenkov cpm), and their binding affinity and specificity was assessed by gel shift analysis. Lanes 1 through 10 represent molar ratios of PNA_{PBS} to U5 PBS RNA of 0, 0.1, 0.3, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, and 5.0, respectively. Lanes 11–15 represent gel shift with PNA_{SCB} at molar ratios of PNA_{SCB} to U5 PBS RNA of 0.1, 0.5, 1.0, 2.5, and 5.0, respectively. The labeled U5 PBS RNA band was quantified on phosphorImager using Image-Quant software (Molecular Dynamics). The percentage of U5 PBS RNA retarded due to PNA binding is as indicated under each lane.

clearly demonstrate the efficacy of PNA_{PBS} in preventing the sssDNA synthesis on $tRNA_3^{Lys}$ primer (lanes 2–7). At as low as 2.5 molar ratio of PNA_{PBS} to TP, near complete absence of ssDNA product was noted (lane 2). The nonspecific scrambled PNA exhibited no effect on reverse transcription under identical conditions (Fig. 4B, lanes 8–10). These results suggest that PNA_{PBS} once bound to its target sequence cannot be competed out by $tRNA_3^{Lys}$.

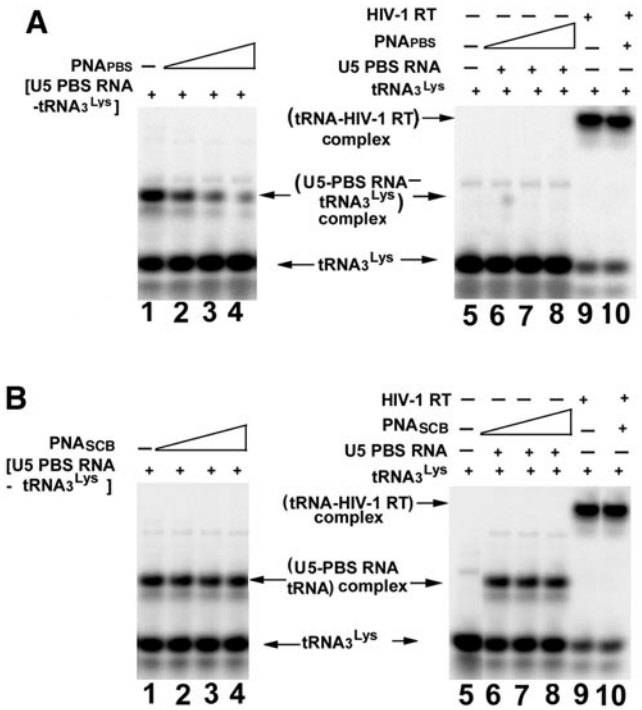

FIG. 3. Effect of PNA_{PBS} or PNA_{SCB} on the formation of ($tRNA_3^{Lys}$-U5 PBS RNA) complex. U5 PBS RNA preprimed with the labeled $tRNA_3^{Lys}$ was incubated in the presence or absence of PNA_{PBS} (A) or PNA_{SCB} (B) under conditions as stated for the individual lanes. The complex formed was resolved on a native gel and analyzed on a phosphorImager. Lanes 1–4, incubation of the preformed complex in the presence of PNA_{PBS} or PNA_{SCB} at molar ratios of PNA to U5 PBS RNA of 0.0, 1.0, 2.5, and 5.0, respectively. Lane 5, ^{32}P-labeled $tRNA_3^{Lys}$ alone; lanes 6–8, U5 PBS RNA was preincubated with PNA_{PBS} or PNA_{SCB} at molar ratios of PNA to U5 PBS RNA of 1.0, 2.5, and 5.0, respectively, followed by further incubation with labeled $tRNA_3^{Lys}$; Lanes 9 and 10, represent formation of ($tRNA_3^{Lys}$-HIV-1 RT) complex in the absence or presence of 1 μM PNA_{PBS} or PNA_{SCB}, respectively. The positions of free $tRNA_3^{Lys}$, (U5 PBS RNA-$tRNA_3^{Lys}$) complex, and ($tRNA_3^{Lys}$-HIV-1 RT) complex are as indicated.

PNA_{PBS} mediated inhibition of the endogenous reverse transcription in the disrupted HIV-1 virions

Since PNA_{PBS} significantly inhibited the *in vitro* initiation and subsequent extension of $tRNA_3^{Lys}$-primed reverse transcription, it was of interest to see whether such inhibition could also be seen with the isolated HIV-1 virion particles. We therefore examined the efficacy of PNA_{PBS} in invading the $tRNA_3^{Lys}$-primed duplex region in the disrupted HIV-1 virions and blocking the process of reverse transcription. Aliquots of disrupted virions were incubated in the presence of varying concentrations of PNA_{PBS} or scrambled PNA for a stated time. Reverse transcription was then catalyzed by the endogenous HIV-1 RT present in the disrupted virions, by supplementing the reaction mixture with a cocktail of dNTPs including labeled dCTP. The result of this analysis is presented in Fig. 5A. In the absence of PNA_{PBS} (lane C), substantial amounts of reverse-transcription products, smaller as well as larger than the expected size of (−) sssDNA (257 nucleotide), were obtained. The presence of larger products may be attributed to the efficient strand transfer reaction, whereas incomplete (−) sssDNA products may have accumulated due to their inability to participate in the strand transfer reaction. Incubation of the disrupted virions with PNA_{PBS} significantly decreased the reverse-transcription products. This decrease corresponded to both the initiation and the subsequent elongation products and correlated with the concentration of PNA_{PBS} (lanes 1–8). At 25 nM PNA_{PBS} concentration, approximately 30% inhibition of reverse transcription was obtained (lane 2), which gradually increased to 95% inhibition at 750 nM PNA_{PBS} concentration (lane 6). In contrast,

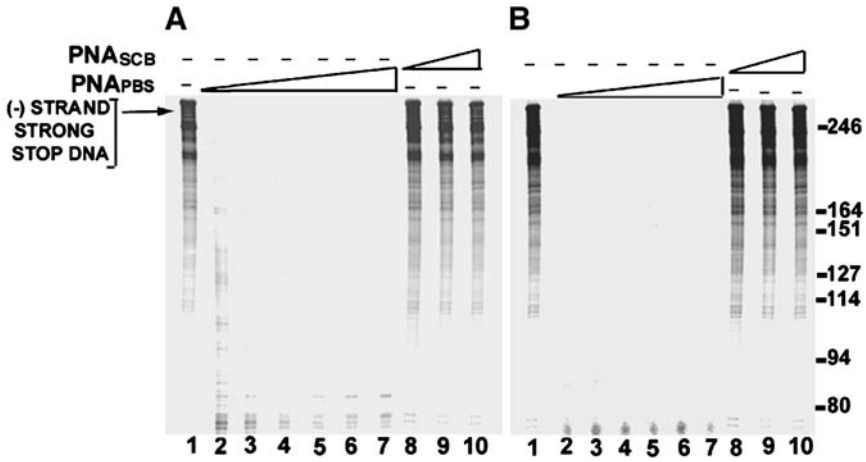

FIG. 4. Effect of PNA_{PBS} on $tRNA_3^{Lys}$-primer extension assay. The $tRNA_3^{Lys}$-primer was annealed to U5 PBS RNA and incubated in the absence or presence of varying concentrations of PNA_{PBS} or PNA_{SCB} (A). In another set U5 PBS RNA was preincubated in the absence or presence of varying concentrations of PNA_{PBS} or PNA_{SCB} followed by priming with $tRNA_3^{Lys}$ (B). Reverse transcription was initiated by the addition of HIV-1 RT, dNTPs, and Mg^{2+}. The reaction products were resolved on a denaturing polyacrylamide–urea gel and analyzed on a phosphorImager. Lane 1, control without PNA; Lanes 2 through 7 show the reverse transcription products in the presence of PNA_{PBS} at 2.5, 5.0, 7.0, 8.0, 9.0, and 10.0 molar ratios of PNA_{PBS} to TP, respectively. Lanes 8 through 10 represent the reactions carried out in the presence of PNA_{SCB} at 2.5, 7.0, and 10.0 molar ratio of PNA_{SCB} to TP, respectively. The $tRNA_3^{Lys}$-derived (−) sssDNA product corresponded to the predicted size of 257 nucleotides. The position of the molecular markers is indicated to the right.

incubation of the disrupted virions with scrambled PNA (5 μM) did not influence the endogenous reverse-transcription process (lane marked S), thus indicating the specificity of PNA_{PBS} for its target sequence.

In another set, we examined the efficacy of PNA_{PBS} in blocking the process of reverse transcription on purified vRNA isolated from HIV-1 virions. The tRNA primer remains bound to the viral genomic RNA upon isolation and can be visualized in a tRNA-primer extension assay upon addition of RT enzyme and dNTPs (Fig. 5B). Unlike in the case of disrupted virion particles, tRNA extension on the purified vRNA yielded a prominent 257-nt-long tRNA–cDNA product (lane C). We established the identity of this 257-nt-long tRNA–cDNA product by NaOH-mediated degradation of the tRNA part, leaving a 181-nt cDNA product (results not shown). Incubation with increasing concentrations of PNA_{PBS} in the reaction mixture resulted in a corresponding decrease of the reverse transcription product (lanes 1–8), thus indicating the efficacy of PNA_{PBS} in displacing the bound tRNA from its complementary PBS sequence on the HIV-1 viral RNA and disrupting reverse transcription. Further, the specificity of interaction of PNA_{PBS} in targeting its corresponding sequence was indicated by the observation that scrambled PNA had no effect on the reverse-transcription process (lane S).

Inhibition of HIV-1 replication in CEM cells by anti-PBS PNA

Since anti-PBS PNA inhibited the reverse-transcription process in the disrupted virions, we investigated their efficacy in HIV-1-infected cell cultures. T cell lymphocytes, infected with the pseudotyped HIV-1 virus carrying the firefly luciferase reporter, were transfected with varying concentrations of the individual anti-PBS PNA or scrambled PNA. Cell extracts were then normalized for the total protein and analyzed for quantitative levels of luciferase expression to evaluate the effect of varying concentrations of PNA_{PBS} on HIV-1 production in these cells. Luciferase activity obtained in the infected T cells in the absence of PNA was arbitrarily set at 100% and that obtained in the presence of PNA was calculated relative to this value. These results presented in Fig. 6 represent the percentage luciferase activity at indicated concentrations of PNA_{PBS} in CEM (Fig. 6A) and Jurkat cells (Fig. 6B). Substantial inhibition in virus production as indicated by a corresponding decrease in the luciferase activity was seen in both the T cell types. The extent of inhibition directly correlated with the concentrations of PNA_{PBS}. At 0.5 μg PNA_{PBS} concentration, a modest inhibition of approximately 12–14% was obtained, which increased to 54–58% inhibition at 1 μg PNA_{PBS}. A near abolishment of virus production (approximately 1–2% luciferase activity) was obtained at 10 μg of PNA_{PBS}. By contrast, scrambled PNA at similar concentration exhibited no inhibition of virus production, thus indicating the specificity of PNA_{PBS}-mediated inhibition. These results clearly demonstrate the antiviral efficacy of PNA_{PBS}.

Mechanism of PNA_{PBS}-mediated inhibition of virus production

A substantial reduction in the expression of the luciferase reporter in both the CEM and the Jurkat cells suggested inhibition of transcription of the HIV-1 mRNA.

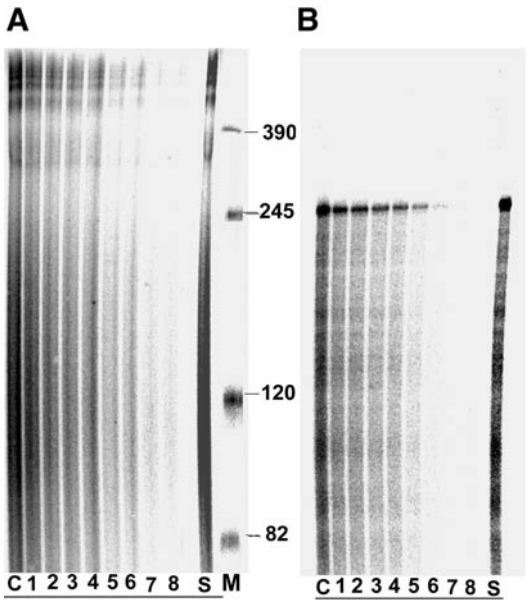

FIG. 5. PNA_{PBS}-mediated inhibition of reverse transcription in disrupted HIV-1 virions (A) and purified vRNA-tRNA (B). To examine the efficacy of PNA_{PBS} in invading the $tRNA_3^{Lys}$ primed duplex region and blocking the process of reverse transcription, varying concentrations of PNA_{PBS} or PNA_{SCB} were incubated with disrupted HIV-1 virions containing the endogenous $tRNA_3^{Lys}$-primed viral RNA genome, reverse transcriptase and other viral proteins (A) or with purified viral RNA associated tRNA (B). Reverse transcription in the disrupted virions was carried out by endogenous RT upon supplementation with dNTP-Mg^{2+}. A similar set of reactions was carried out in the case of the isolated HIV-1 RNA–tRNA incubation mix, except that extraneous HIV-1 RT was included in the assay. The reaction products were resolved on a denaturing polyacrylamide–urea gel and analyzed by phosphorImaging. Lane C represents reverse transcription products obtained in the absence of PNA; Lanes 1 through 8 represent reactions carried out at PNA_{PBS} concentrations of 25 nM, 50 nM, 100 nM, 250 nM, 500 nM, 750 nM, 1 μM, and 5 μM, respectively. Lane S represents reverse transcription product obtained in the presence of 5 μM PNA_{SCB}. Position of the molecular markers is shown in the lane marked M. The (−) ssDNA product obtained with the isolated vRNA–tRNA complex corresponded to the expected size of 257 nucleotides. This product, in the case of the disrupted virions, was larger than 257 nucleotides, possibly representing products obtained due to strand transfer reaction.

It is possible that the decreased transcription (results not shown) may be due to lower levels of integrated HIV-1 cDNA into host cell genome. A decrease in cDNA synthesis would be a direct consequence of PNA_{PBS} interference in the reverse-transcription process as observed in tRNA primer extension analysis *in vitro* (Fig. 4), as well as in disrupted HIV-1 virions (Fig. 5A). We therefore analyzed the amounts of integrated HIV-1 cDNA in the host cell genome. For this, molar excess of oligomeric PBS primer was primed with 1 μg of total DNA isolated from the PNA_{PBS} transfected, infected T cell lymphocytes. The annealed PBS primer was extended using radiolabeled nucleotides and HIV-1 RT and products were resolved on an agarose gel and analyzed by phosphorimaging. The results are presented in Fig. 7. Extension of the annealed PBS primer on the integrated viral DNA in the host DNA resulted in a prominent product corresponding to approximately 2–2.5 kb in size (lanes 2–9). A complete absence of product in the uninfected cells (lane 1) established the identity of this 2- to 2.5-kb product as a result of PBS primer extension after annealing specifically to its complementary sequence on the integrated viral DNA. The intensity of this product decreased quantitatively with increasing PNA_{PBS} concentrations (lanes 3–8) in both CEM and Jurkat cells, thus indicating that the population of viral DNA present in total DNA decreased with increasing PBS concentration. Interestingly, scrambled PNA did not affect the amount of product synthesis (lane 9). These results indicate that the mechanism of PNA_{PBS} inhibition may be directly related to interference at the step of reverse transcription.

DISCUSSION

During the assembly of the HIV-1 virions, several cellular tRNAs including $tRNA_3^{Lys}$ are packaged into the

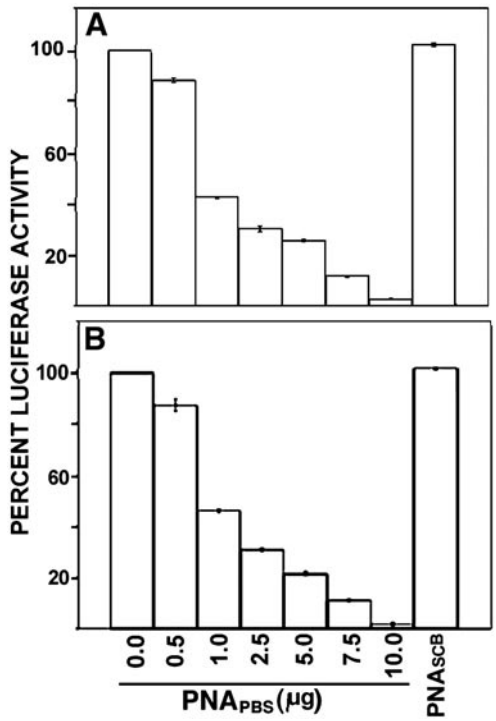

FIG. 6. PNA_{PBS} inhibits HIV-1 replication in T cells. T cell lymphocytes infected with the pseudotyped HIV-1 virus expressing the firefly luciferase reporter were transfected with indicated amounts of PNA_{PBS} or nonspecific scrambled PNA (PNA_{SCB}). The effect of PNA_{PBS} on HIV-1 replication was monitored by analyzing the expression of firefly luciferase in the cell extracts, at 48 h posttransfection. Firefly luciferase activity was normalized to the total protein in the cell extract. Luciferase activity obtained with HIV-1-infected CEM or Jurkat cells, transfected with PNA_{PBS} at indicated concentrations, are shown in A and B, respectively. The amounts of PNA_{SCB} transfected corresponds to 10 μg. Luciferase expression obtained in the absence of PNA in the mock-transfected HIV-1 infected T cells was arbitrarily set at 100% and luciferase expression in the presence of PNA was calculated relative to this value. The values shown are an average of three sets of experiments. The bar represents standard deviation.

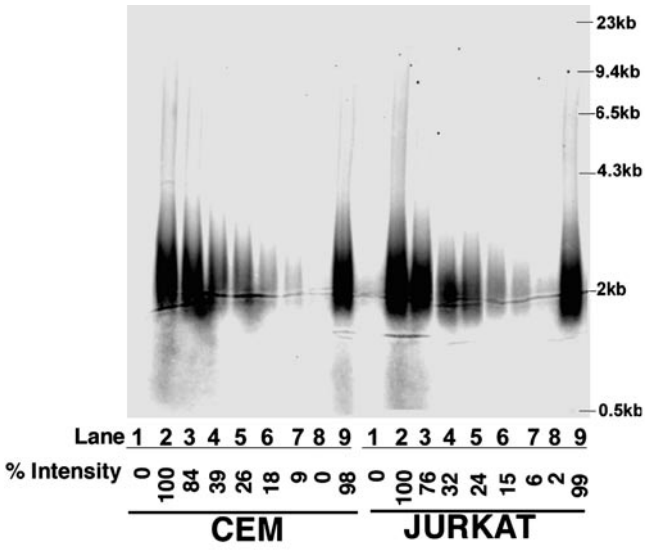

FIG. 7. Quantitation of integrated HIV-1 cDNA. HIV-1 cDNA population integrated into the host genomic DNA was estimated from total DNA isolated from HIV-1-infected T cell lymphocytes, transfected with varying amounts of PNA_{PBS} or PNA_{SCB}. One microgram of total DNA was hybridized with the 17-mer PBS primer, complementary to the primer binding sequence (nucleotides 183–201 of the HIV-1 genomic RNA) and extended with HIV-1 RT in the presence of dNTPs and Mg^{2+}. The products were resolved on agarose gel; the gel was dried and analyzed on a phosphorImager. A representative figure of one such analysis is presented. Lane 1 shows absence of product in uninfected cells. Lanes 2 through 8 represent DNA products obtained at 0, 0.5, 1.0, 2.5, 5.0, 7.5, and 10 μg of PNA_{PBS} concentration. Lane 9 exhibits similar intensity of product in PNA_{SCB}-transfected CEM or Jurkat cells. The size of this product is approximately 2–2.5 kb. The position of the lambda *Hin*dIII-digested markers in indicated on the right. The intensity of the product obtained was quantified using ImageQuant software (Molecular Dynamics). The value obtained in the absence of PNA (Lane 2) was arbitrarily set at 100% and that obtained in the presence of PNA was calculated relative to this value. These values are indicated at the bottom of each lane.

virion particles, but HIV-1 utilizes only $tRNA_3^{Lys}$ for the initiation of reverse transcription (Jiang *et al.,* 1993). The $tRNA_3^{Lys}$ primes to the PBS, an 18-nucleotide sequence which is located downstream of the U5 region of the 5′ LTR and spans from nucleotides 183–201 of the viral RNA genome. The 3′-terminal 18 nucleotides of the primer $tRNA_3^{Lys}$ are complementary to the PBS region (Ratner *et al.,* 1985). HIV-1 mutants that use reverse-transcription primers other than the natural $tRNA_3^{Lys}$ exhibit reduced replication efficiencies and revert to the wild-exhibit-type $tRNA_3^{Lys}$ sequence upon prolonged culturing (Das *et al.,* 1995). Apart from the interaction of $tRNA_3^{Lys}$ with the PBS, the A-loop region located upstream of the PBS has been implicated in the selection and stabilization of $tRNA_3^{Lys}$ on the viral genome (Li *et al.,* 1997). This suggests the importance of the primer-binding sequence as well as the A-loop sequence during viral replication.

In the present study, we demonstrate the potential of PNA complementary to the 5 nucleotides of the PBS and 11 nucleotides of the A-loop region (PNA_{PBS}) to inhibit virus replication in cell culture. PNA_{PBS} stoichiometrically binds to the PBS/A-loop region of the U5 PBS RNA (Fig. 2) and efficiently displaces the bound $tRNA_3^{Lys}$ primer (Fig. 3A). By contrast, scrambled PNA (Fig. 3B) as well as an oligonucleotide having identical sequence as PNA_{PBS} (results not shown) were unable to disrupt the preformed ($tRNA_3^{Lys}$-U5 PBS RNA) complex. This suggests that unlike an oligonucleotide, the uncharged PNA_{PBS} is able to invade the duplex region of $tRNA_3^{Lys}$-primed viral RNA genome and disrupt this interaction. Our findings that the binding of HIV-1 RT to the $tRNA_3^{Lys}$ alone is not affected by PNA_{PBS}, further suggesting that the affinity of PNA_{PBS} is highly specific toward its target sequence on the viral genome (Fig. 3A, lanes 9 and 10).

It is possible that sequestering of the PBS region of the viral genome by the complementary PNA_{PBS} may have a strong destabilization effect on the formation of enzyme-$tRNA_3^{Lys}$–viral RNA ternary complex. This interaction may, in turn, prevent initiation of HIV-1 reverse transcription and transition of the initiated primer to elongation. We therefore investigated *in vitro* the effect of PNA_{PBS} on the natural $tRNA_3^{Lys}$-primed initiation of reverse transcription catalyzed by HIV-1 RT. In the absence of PNA_{PBS} or with scrambled PNA, the $tRNA_3^{Lys}$-derived reverse transcription yielded a full-length (−)-strand strong stop DNA product of predicted size (257 nucleotides). Incubation of PNA_{PBS} with U5 PBS RNA preprimed with $tRNA_3^{Lys}$ resulted in significant reduction in the initiation of reverse transcription and its subsequent elongation (Fig. 4A; lanes 2–7). This suggests the ability of PNA_{PBS} in destabilizing the $tRNA_3^{Lys}$ primer by invading the duplex region of the viral RNA. The inhibitory effect of PNA_{PBS} was more pronounced when incubated with unprimed U5 PBS RNA (Fig. 4B; lanes 2–7), suggesting that PNA_{PBS} once bound to its target can effectively interfere with the priming process.

It was therefore interesting to determine whether PNA_{PBS} could inhibit the process of reverse transcription in the disrupted virions containing the endogenous $tRNA_3^{Lys}$-primed viral RNA genome and other viral components/proteins. Our results indicate that incubation of the disrupted virions with PNA_{PBS} distinctly inhibited the reverse-transcription process as a function of PNA_{PBS} concentration (Fig. 5A; lanes 1–8), thus indicating the ability of PNA_{PBS} to sequester its target in the disrupted virions. Interestingly, purified vRNA–tRNA complex isolated from HIV-1 virions yielded the characteristic 257-nucleotide product (Fig. 5B, lane C), while the disrupted virions yielded products longer than 257 nucleotides (Fig. 5A). This suggests that the longer products with the disrupted virions may have arisen as a result of strand transfer.

These results imply that interaction of PNA_{PBS} with the PBS sequences on the viral genome blocks the base-pairing interaction between PBS and the 3′ terminal 5 nucleotides of $tRNA_3^{Lys}$, resulting in destabilization of the

$tRNA_3^{Lys}$ primer on the viral genome. Modification from SUU to the suppressor anticodon CUA in the anticodon of $tRNA_3^{Lys}$ also destabilizes the interaction between *in vivo* packaged $tRNA_3^{Lys}$ and the viral RNA genome (Huang *et al.,* 1996). The inability of *in vivo* packaged mutant $tRNA_3^{Lys}$ (CUU) to prime reverse transcription was suggested to be a consequence of its inability to interact with this A-rich loop of the viral genome. HIV-1 containing the PBS complementary to $tRNA^{His}$ rather than $tRNA_3^{Lys}$ quickly reverts to the original PBS sequence complementary to $tRNA_3^{Lys}$. However, when the A-loop was changed such that it was complementary to the $tRNA^{His}$ anticodon, the $tRNA^{His}$ became stabilized in the viral population (Wakefield *et al.,* 1996). It is thus obvious that integrity of both the PBS region and the A-loop structure is essential for viral infectivity.

Interestingly, all the natural occurring HIV-1 strains isolated to date from clinical samples harbor the PBS complementary to the $tRNA_3^{Lys}$. Our results indicate that PNA_{PBS} can inhibit HIV-1 replication in cell culture (Fig. 6). Our observation, that the amounts of proviral DNA integrated in the host DNA decrease at increasing PNA_{PBS} concentration (Fig. 7), strongly suggest that the mechanism of PNA_{PBS}-mediated interference may be regulated at the level of reverse transcription, though we cannot rule out its ability to interfere at the step of transcription directly by sequestering the PBS sequence of the nascent RNA (results not shown).

The efficacy of PNA oligomers over conventional DNA or RNA antisense oligonucleotides may be attributed to a number of factors. PNA exhibit high affinity and specificity for their complementary DNA and RNA sequences (Eghlom *et al.,* 1993), unlike oligonucleotides, which are often confounded by nonspecific interactions (Stein, 1999). Moreover, PNA exhibit an added advantage in that they can invade the duplex DNA/RNA by invasion or displacement of one of the DNA strands and bind to their target site (Lee *et al.,* 1997; Peffer *et al.,* 1993; Mollegaard *et al.,* 1994). These molecules are also chemically stable and resistant to cleavage by nucleases and proteases (Demidov *et al.,* 1994).

It has been shown that PNA targeted to the coding region of the Ha-ras mRNA effectively blocked the formation of the translation initiation complex and polypeptide chain elongation (Dias *et al.,* 1999). Likewise, PNA targeted to the telomerase RNA blocked its activity, resulting in progressive telomere shortening and causing immortal human breast epithelial cells to undergo apoptosis (Shammas *et al.,* 1999; Hamilton *et al.,* 1999). Removal of antitelomerase PNA from the cell culture restored the telomere length (Herbert *et al.,* 1999). The Bcl-2 protein synthesis in cell-free system was effectively blocked by PNA targeted to AUG start codon and 5′ UTR of human B cell lymphoma (Bcl)-2 gene (Molongi *et al.,* 1999). A significant inhibition of the antinociceptive and locomotor response to the delta opoid receptor agonists was noted upon repeated i.c.v. administration of PNA targeted to a region of the rat delta opoid receptor (Fraser *et al.,* 2000).

Recently, several studies involving PNA conjugated to certain transporter peptides have been shown to be efficiently taken up by the cells and to exhibit antisense activity on the targeted gene (Chinnery *et al.,* 2000; Kamagai *et al.,* 2000; Ljungstrom *et al.,* 1999; Branden *et al.,* 1999; Harrison *et al.,* 1999; Scarfi *et al.,* 1999; Aldrian-Herrada *et al.,* 1998; Pooga *et al.,* 1998). The current focus of our studies is to improve the biodelivery of PNA_{PBS} in the cells by conjugating it with membrane-transducing peptides (MTD). Using this approach, we have recently demonstrated the uptake efficiency and antiviral efficacy of anti-TAR PNA conjugated with transportan MTD vector (Kaushik *et al.,* 2002). Studies are underway to address the biodelivery and antiviral efficacy of PNA_{PBS} conjugated with various MTD vectors.

MATERIALS AND METHODS

DNA modifying enzymes were purchased from Roche Molecular Biochemicals. HPLC-purified human placental $tRNA_3^{Lys}$ was obtained from BIO S&T (Canada). Total DNA isolation kit (Qiamp DNA kit) was a product of Qiagen Inc. Cell culture media, fetal bovine serum (FBS), and transfection reagents were either from Life Technologies or Roche Molecular Biochemicals. Luciferase assay kit was from Promega. Tritiated dNTPs, γ-^{32}P-ATP, and α-^{32}P-dNTPs were the products of Perkin–Elmer Life Sciences. Pore-size filters measuring 0.45 μm were from Schleicher & Schuell. ELISA p24 antigen kit was from Abbott Laboratories. DNA oligomers were synthesized at the Molecular Resource Facility at UMDNJ. Polyamide nucleic acid oligomers were synthesized at Applied Biosystems Inc. All other reagents were of the highest available purity grade and purchased from Fisher.

Plasmid and clones

The wild-type homodimeric (p66/p66) HIV-1 RT was purified using the expression vector, pKK-RT66, constructed in this laboratory (Lee *et al.,* 1997). An HIV-RNA expression clone pHIV-PBS was a generous gift from Dr. M. A. Wainberg (Arts *et al.,* 1994). This clone contains a 947-bp fragment (+473 to +1420) of pHλHXB2 HIV-1 proviral clone and was used to generate RNA transcript corresponding to the U5 PBS region. Another HIV-1 RNA expression clone, pU5-PBS constructed in this laboratory (Kaushik *et al.,* 2001), was used to transcribe shorter HIV-1 RNA (200 nucleotide) for gel-shift analysis. This clone contains a 183-bp fragment corresponding to nucleotides 473–656 of pHλHXB2 HIV-1 proviral clone comprising PBS, U5, and part of the R region. An additional 17 nucleotides flanking the 5′ terminus are derived from the plasmid vector. The plasmid, pVSV-G, encoding the vesicular stomatitis virus protein G under the control of the

CMV immediate-early promoter was purchased from BD Biosciences Clontech. The plasmid, pHIV-$_{1JR\text{-}CSF\text{-}luc}$env(−), was a kind gift from Dr. Planelles (Planelles *et al.,* 1995). This replication-defective HIV-1 clone lacks the envelope gene and has a firefly luciferase reporter cloned in place of nef in the HIV-$_{1JR\text{-}CSF}$env(−) cassette.

Isolation of p66/51 HIV-1 RT

The recombinant clone pKK-RT66 encoding the wild-type p66 HIV-1 RT was expressed in JM109 and purified as described before (Lee *et al.,* 1997). To generate the heterodimeric HIV-1 RT (p66/p51), the homodimeric enzyme (p66/p66) was subjected to proteolytic cleavage by HIV-1 protease as follows: The p66/p66 species of HIV-1 RT was incubated with HIV-1 protease at 1:1 molar ratio in a buffer containing 0.1 M potassium phosphate, pH 7.5, 1.0 M NaCl, 1 mM DTT, and 1 mM EDTA. Following 16 h incubation at 4°C, the extent of proteolytic cleavage was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Coomassie blue staining. The heterodimeric enzyme was isolated on a phosphocellulose column (Lee *et al.,* 1997) and the final enzyme preparation was stored at −70°C in a buffer containing 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 1 mM DTT, and 50% glycerol. Protein concentrations were determined by using the Bio-Rad colorimetric kit as well as by spectrophotometric measurements using $É_{280} = 2.62 \times 10^5\ M^{-1}\ cm^{-1}$ for p66/51 heterodimer.

Preparation of ^{32}P-labeled U5 PBS RNA template

The plasmid pU5-PBS was used to transcribe labeled RNA for assessing the binding affinity of PNA$_{PBS}$ to its target sequence on the HIV-1 genomic RNA, as described previously (Kaushik *et al.,* 2001). Briefly, the plasmid was linearized with the restriction enzyme *Xho*1 and internally labeled with α-^{32}P-UTP (3000 Ci/mmol; Amersham Life Sciences) using T7 RNA polymerase (Roche Molecular Biochemicals). The plasmid was digested with DNase I free of RNase and the labeled transcript was extracted with phenol-chloroform and alcohol precipitated. The RNA product was dissolved in DEPC-treated water containing 10 mM DTT, further purified by G-50 spin column, and stored at −70°C.

Gel retardation assay

The ^{32}P-labeled U5 PBS RNA (5×10^3 Cerenkov cpm) was incubated with PNA$_{PBS}$ or scrambled PNA at varying molar ratios for 3 h at 37°C in a binding buffer containing 50 mM Tris–HCl, pH 7.8, 60 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, 10% glycerol, 0.01% bovine serum albumin, 0.01% NP-40, and 500 ng of poly[r(I-C)], in a final volume of 15 μl. Three microliters of RNA gel-loading dye (0.27% bromphenol blue and 20% glycerol) was added to the samples and subjected to non-denaturing gel analysis on a 8% polyacrylamide gel in Tris–borate–EDTA (TBE) buffer, pH 8.2. The gels were routinely prerun at 100 V for 30 min at 4°C in TBE buffer. The (U5 PBS RNA–PNA) complexes were resolved at a constant voltage of 150 V at 4°C. The gel was dried and analyzed on a phosphorimager (Molecular Dynamics). The percentage of labeled U5 PBS RNA retarded as a result of PNA binding was quantified by Image-Quant.

Labeling of tRNA$_3^{Lys}$

The HPLC purified human placental tRNA$_3^{Lys}$ obtained from BIO S&T was 3′ end-labeled with [^{32}P]pCp by T4 RNA ligase (Ausubel *et al.,* 1998). The labeled product was extracted three times with phenol chloroform, precipitated with alcohol, lyophilized, and suspended in TE buffer. This was further purified on a NAP-10 column to remove the unincorporated radiolabeled nucleotides.

Preparation of R-U5-PBS HIV-1 RNA template

The plasmid, pHIV-PBS, was used to transcribe the 495-base-long U5-PBS RNA template as described earlier (Arts *et al.,* 1994). Briefly, the plasmid pHIV-PBS was linearized with the restriction enzyme Acc1 and transcribed using T7 RNA polymerase and other reaction components from Roche Biochemicals. After *in vitro* transcription reaction, the DNA template was removed by DNase I digestion, followed by phenol-chloroform extraction and alcohol precipitation. The RNA products were dissolved in DEPC-treated water containing 10 mM DTT, further purified by G-50 spin column, and stored at −70°C.

Effect of PNA$_{PBS}$ on the formation of (tRNA$_3^{Lys}$-U5 PBS RNA) complex

U5 PBS RNA was preprimed with labeled tRNA$_3^{Lys}$ (7.5×10^3 Cerenkov cpm) and incubated at 37°C for 2 h with PNA$_{PBS}$ or scrambled PNA at varying molar ratios of PNA$_{PBS}$ to U5 PBS RNA. The incubation buffer contained 50 mM Tris–HCl, pH 7.8, 10 mM DTT, 0.01% BSA, 60 mM KCl, and 5 mM $MgCl_2$. In another set, U5 PBS RNA was preincubated with PNA$_{PBS}$ or scrambled PNA at 37°C for 1 h and then supplemented with the labeled tRNA$_3^{Lys}$ and further incubated for 2 h. In a separate set, 1 μM HIV-1 RT was incubated with labeled tRNA$_3^{Lys}$ (7.5×10^3 Cerenkov cpm) in the absence or presence of PNA$_{PBS}$ or scrambled PNA. Three microliters of RNA gel loading dye was added to 15 μL of the reaction mixture. The (U5-PBS RNA–tRNA$_3^{Lys}$) complex formed was resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel at 150 V in TBE buffer. The gels were dried and analyzed on a phosphorimager.

Reverse transcription of U5-PBS RNA template primed with natural tRNA$_3^{Lys}$

U5 PBS RNA was annealed with the natural tRNA$_3^{Lys}$ at 2:1 molar ratio. Reverse-transcription reactions were car-

ried out by incubating 5 nM of U5 PBS RNA/$tRNA_3^{Lys}$ template primer with 50 nM HIV-1 RT, in a reaction mixture containing 50 mM Tris–HCl, pH 7.8, 10 mM DTT, 100 μg/mL BSA, 5 mM $MgCl_2$, and 50 μM each of dATP, dTTP, dGTP, and 2 μM of α-^{32}P-dCTP (0.5 μCi/pmol). Reactions were initiated by the addition of enzyme and terminated by the addition of 20 mM EDTA. The samples were extracted with phenol-chloroform, precipitated with alcohol, and lyophilized. Ten microliters of 1× Sanger's gel loading solution was added to the samples (Sanger *et al.,* 1977) and the products were resolved on an 8% polyacrylamide–urea gel.

The effect of PNA_{PBS} on *in vitro* natural $tRNA_3^{Lys}$-primed reverse transcription was assessed as follows. In one set, varying molar ratios of PNA_{PBS} or scrambled PNA was incubated with U5 PBS-RNA template preprimed with the $tRNA_3^{Lys}$ at 37°C for 2 h. In another set, PNA_{PBS} or scrambled PNA was first incubated with U5 PBS–RNA template at 37°C for 2 h, followed by priming with $tRNA_3^{Lys}$. Reverse transcription on both these sets was assessed as described above.

Cell culture and transfection

CEM and Jurkat T cell lymphocytes were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 4 mM L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C and 5% CO_2. The chronically HIV-1-infected H9 cells ($H9_{Lai}$) were maintained under identical conditions except that they were supplemented with 20% fetal calf serum. Human 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. For transfection in CEM, the cells were grown to mid-log phase, washed with phosphate-buffered saline without Ca^{+2} or Mg^{+2}, resuspended in RPMI-1640 medium (5.0×10^6 cells in 250 μl), and electroporated at 250 V/900 microfarad capacitance using the Bio-Rad Gene pulsar II. Jurkat and 293T cells were transfected in accordance with the manufacturer's protocol using the X-tremeGENE Q2 Transfection reagent (Roche Molecular Biochemicals) or the calcium-phosphate transfection system (Life Technologies), respectively.

Isolation of HIV-1 virions

$H9_{LAI}$ cells were used as the source of the HIV-1 virus. These chronically HIV-1-infected cells were extensively washed to remove previously produced virions, suspended in 25 ml of RPMI 1640 complete medium at a cell density of 10^4 cells/ml, and grown for 3 days in a 37°C incubator. The culture medium was centrifuged at 1200 rpm for 7 min to remove cells. An aliquot of the cell supernatant was used for p24 antigen estimation. The virus-containing supernatant was filtered through a 0.45-μm pore-size filter (Schleicher & Schuell), and the virions were pelleted by centrifugation at 28,000 rpm for 45 min in a Beckman SW28 rotor. The HIV-1 virion pellet was disrupted in 300 μl buffer containing 50 mM Tris–HCl, pH 7.8, 100 mM KCl, 0.5 mM EDTA, and 0.2% NP-40 and used as a source of $tRNA_3^{Lys}$-primed HIV-1 viral RNA genome, endogenous RT, and other packaged viral components.

Isolation of HIV-1 vRNA

HIV-1 virions isolated from $H9_{LAI}$ cells as described above were used as a source for extracting the virion RNA. The virions were resuspended in 500 μl of buffer containing 50 mM Tris–HCl (pH 7.8), 100 mM NaCl, 2.5 mM EDTA, 1.0% SDS, and 300 μg of proteinase K and incubated at 37°C for 30 min, followed by 3× extraction with phenol-chloroform-isoamyl alcohol (25:24:1). RNA was precipitated in 0.3 M Na-acetate (pH 5.2) and 70% ethanol at −80°C, centrifuged at 16,000 *g* for 30 min, washed with 70% ethanol, and air dried. Contaminating DNA was removed by digestion with DNase I (RNase-free; Roche Biochemicals) as per the supplier's instructions. RNA was reextracted with phenol-chloroform-isoamyl alcohol (25:24:1), precipitated with 0.3 M Na acetate and 70% ethanol, and pelleted at 16,000 *g* for 30 min. Following 70% ethanol washing, the air-dried RNA pellet was resuspended in 100 μl of 25 mM Tris–HCl, pH 7.8/0.5 mM EDTA, and stored at −20°C.

Endogenous reverse transcription in the disrupted HIV-1 virions and tRNA-primer extensions on associated viral RNA in the presence of PNA_{PBS}

The effect of PNA_{PBS} on endogenous reverse transcription in the disrupted HIV-1 virions as well as on viral RNA-associated tRNA was assessed as follows: Disrupted HIV-1 virions (10 μl) were incubated at 4°C for 3 h and subsequently at 37°C for 30 min, in the presence or absence of varying concentrations of PNA_{PBS} or scrambled PNA in a 15 μl volume. The components of the incubation mix were 50 mM Tris–HCl, pH 7.8, 10 mM DTT, 66 mM KCl, 0.3 mM EDTA, 0.14% NP-40, and 1 U/μl RNasin. Reverse transcription was initiated by the addition of 5 μl of 4× RT buffer (40 mM $MgCl_2$, 40 mM dithiothreitol, 200 mM KCl, 120 μg of actinomycin D/ml, 100 μg BSA/mL, 40 μM each of dATP, dGTP, dTTP, 4 μM dCTP), and 5 μCi of α-^{32}P-dCTP (3000 Ci/mmol). The labeled cDNA product generated at 37°C for 5 min was further chased for 30 min by the addition of 1 μl deoxynucleoside triphosphate (dNTP) mix (10 mM each dNTP) and the reaction was quenched with 50 mM EDTA. Following phenol–chloroform extraction and alcohol precipitation, the pellet was resuspended in formamide loading buffer, heated at 90°C for 5 min, resolved on a denaturing 6% polyacrylamide–urea gel, and analyzed by phosphorimaging.

In another set, similar reactions were carried out with

the isolated HIV-1 RNA-$tRNA_3^{Lys}$, except that 5 μl of purified HIV-1 RNA-tRNA was used per reaction and 150 ng of recombinant HIV-1 RT was included in the assay.

T cell infections

Pseudotyped HIV-1 virion stocks were produced by cotransfection of pHIV-$_{1JR\text{-}CSF\text{-}luc}$env(−) (Planelles *et al.,* 1995) and pVSV-G (BD Biosciences Clontech) in 293T cells. Culture medium was harvested at 24, 48, and 72 h posttransfection; an aliquot was removed for p24 antigen quantification using the ELISA p24 antigen kit (Abbott Laboratories), and the virus stock was frozen at −80°C. To determine the effect of PNA_{PBS} on HIV-1 production in T cells, CEM and Jurkat cells (5×10^6 cells) were infected at 37°C for 1 h with the pseudotyped HIV-1 virions in the presence of 10 μg/ml of polybrene to achieve multiplicities of infection (m.o.i.) of 10. The cells were extensively washed with PBS to remove the viruses, resuspended in 5.0 ml of complete RPMI medium, and incubated for another 1 h at 37°C. The infected T cells were then transfected in the presence or absence of varying amounts of the individual PNA_{PBS} or scrambled PNA and grown in 10 ml of complete RPMI media. Forty-eight hours posttransfection, the cells were harvested and analyzed for firefly luciferase reporter and HIV-1 cDNA levels as described below.

Luciferase assay

Luciferase assays were performed as per the Promega's luciferase assay protocol. Forty-eight hours posttransfection, the cells were washed with PBS and lysed in 100 μl of the reporter lysis buffer (Promega). The cell extracts were normalized for total protein (Bio-Rad) and assayed for firefly luciferase activity in a 96-well fluorotrac plate using a Packard Top Count Luminescence Counter. The results of at least three separate transfections were analyzed for each experiment.

Total DNA isolation and estimation of integrated HIV-1 cDNA

To estimate the HIV-1 cDNA integrated into the host cell chromosome, total DNA was isolated from the transfected T cells using the Qiamp DNA kit (Qiagen Inc.). A 17-mer PBS oligomer having sequence complementary to the PBS region of the HIV-1 genomic RNA was used as a probe for analyzing the amounts of HIV-1 cDNA integrated into the host genomic DNA by oligomer extension analysis. Hybridization of the probe to the HIV-1 cDNA was achieved by incubating 1 μg of total DNA with 50 pmol of 17-mer PBS oligomer in 1× annealing buffer (50 mM Tris–HCl, pH 7.8 and 50 mM KCl) at 75°C for 7 min and allowing it to cool gradually to room temperature. Extension reactions were carried out in a buffer containing 50 mM Tris–HCl, pH 7.8, 10 mM DTT, 60 mM KCl, 10 mM $MgCl_2$, 100 μg BSA/mL, 10 μM each of dATP, dGTP, dTTP, 1 μM dCTP, 5 μCi of α-^{32}P-dCTP (3000 Ci/mmol) and 200 ng of heterodimeric HIV-1 RT. The labeled cDNA product generated at 37°C for 10 min was chased with cold dNTP mix (500 μM each) for an additional 45 min at 37°C and quenched with 50 mM EDTA. The cDNA product was phenol-chloroform extracted, alcohol precipitated, and the pellet resuspended in 15 μl of 1× loading buffer (10% glycerol and 0.01% each xylene cyanol and bromphenol blue). The products were resolved on 0.8% agarose gel; the gel was dried and subjected to phosphorimager analysis.

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